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GRAM-NEGATIVE BACTERIA:

CHARACTERIZATION OF THE PROTEIN MOIETY ISOLATED BY ACETIC ACID

HYDROLYSIS OF ENDOTOXIN FROM *S. marcescens* 08

W. Wober and P. Alaupovic

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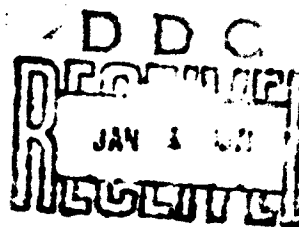University of Oklahoma Medical Center THEMIS Contract

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Abstract

"Conjugated" protein isolated from the endotoxin complex of

Serratia marcescens 08 by 1% acetic acid hydrolysis was char-

acterized by analytical ultracentrifugation, electrophoretic and

immunological properties and chemical analysis. The chemical

composition of "conjugated" protein and other degraded fragments

of endotoxin showed that all characteristic constituents of lipid

A were present exclusively in "conjugated" protein. Successive

treatment of "conjugated" protein with trypsin and pronase resulted

in the isolation of corresponding tryptic and pronase cores which

were characterized by an increased content of lipid A constituents.

Lipid A was separated as an entity from the "conjugated" protein

only after hydrolysis with 0.1 N hydrochloric acid. This result

confirms our previous proposal that lipid A is covalently linked

to the protein moiety in whole endotoxin of S. marcescens 08.

"Conjugated" protein isolated by acetic acid hydrolysis and

"simple" protein prepared by hot aqueous phenol treatment of

whole endotoxin differ from one another not by the presence or

absence of lipid A but rather by the presence of the entire lipid

A moiety in the "conjugated" protein and only a fragment of lipid

A in the "simple" protein.

"Conjugated" protein and its tryptic and pronase cores were

immunogenic and possessed a common antigenic determinant with

"simple" protein and its corresponding cores. Toxicity studies

revealed that both "conjugated" and "simple" proteins retained

to different degrees the toxic properties of whole endotoxin.

Sodium dodecyl sulfate had an enhancing rather than diminishing

effect on the toxicity of endotoxin and its various fragments.

The importance of lipid A as the toxic factor of endotoxins was

established not only indirectly by determining the toxicity of

each fragment containing lipid A, but also directly by demon-

strating that lipid A dispersed in a Tris-SDS buffer displayed

toxic properties similar to those of the lipopolysaccharide

preparation.

Morgan and Partridge (1-3) demonstrated that hydrolysis of

whole endotoxin of Shigella dysenteriae by 1% acetic acid resulted

in the isolation of the so-called "conjugated" protein which may

be converted into "simple" protein by treatment with either 90%

phenol or 0.1 N sodium hydroxide. Because "conjugated" protein

from Shigella paradysenteriae retained the typical toxic properties,

Goebel and his coworkers (4, 5) have referred to protein prepara-

tions isolated by 1% acetic acid hydrolysis as "toxic" proteins.

Since tryptic digestion of the intact endotoxin and of "toxic"

protein did not destroy the toxic component, authors concluded

that the toxin is either not a protein or that it represents a

portion of the protein resistant to the action of proteolytic

enzymes. The suggested existence of a toxic component other

than protein and polysaccharide marked the beginning not only

of an extensive search for but also of a lasting controversy

about the chemical nature of the toxophore group in the endotoxin

complex. Several investigators (6-13) have detected the presence

of a lipid moiety and identified fatty acids and glucosamine as

its principal constituents. However, Westphal and his coworkers

(14-16) were the first to suggest that the firmly bound lipid

moiety (lipid A) was the most probable carrier of typical endo-

toxic effects and, therefore, identical with the active component

of "toxic" or "conjugated" protein. Although the chemical com-

position and structure of "conjugated" protein is not known, it

has been tacitly assumed (17) that the possible presence of lipid

A in "conjugated" protein represents the only difference between

this protein fraction and "simple" protein. To test this assumption,

the physical, chemical and immunological properties of "conjugated"

protein were compared with those of "simple" protein.

MATERIALS AND METHODS

Isolation of the "Conjugated" Protein (C.P.)

from Endotoxin of S. marcescens 08

To remove free lipids, nucleic acid-free endotoxin isolated

by trichloroacetic acid extraction of wet cells of S. marcescens 08

(18) was extracted by chloroform-methanol (2:1, v/v) in a

Soxhlet extractor for 12 hours. About 500 mg of this endotoxin

preparation (LPS-Ud-S) were hydrolyzed by stirring with 250 ml of

preheated 1% acetic acid (Fig. 1) for 4 hours at 90° according

to the method of Morgan and Partridge (1, 2). After cooling for

one hour at 4°, a flocculating, slightly yellow-colored precipitate

was separated from the clear supernate by centrifuging at

17,300 x g for 20 minutes at 10°. The protein preparation was

dissolved in 0.02 M ammonium carbonate buffer, pH 8.6, and after

three successive isoelectric precipitations at pH 3.5 was washed

continuously with distilled water for 24 hours and lyophilized.

The "conjugated" protein was tested for impurities according to

the procedure described for "simple" protein (18). Chloroform

extract of "conjugated" protein contained no free lipids.

After the "conjugated" protein had been separated, the clear

supernate was extracted three times with chloroform to remove

lipids. The lyophilized aqueous phase containing the "degraded"

polysaccharide moiety was dissolved in and dialyzed against

distilled water for 7 days. The outer and inner dialysates were

lyophilized.

Preparation of the Tryptic Core and Pronase Core, and

Isolation of Lipid A from "Conjugated" Protein

The tryptic and pronase cores of "conjugated" protein and

lipid A were obtained according to the procedures utilized for

the preparation of cores and isolation of lipid A from the "simple"

protein (19).

Peptide Mapping

The two-dimensional separation of peptides was carried out

according to the procedure by Katz et al., (20). Tryptic peptides

(2-3 mg) of "simple" proteins or of "conjugated" proteins from

S. marcescens 08 (PX-S) or E. coli O 1^1:K85(B) (PX-E) were

applied to Whatman No. 3 paper and subjected, in the first

dimension, to descending chromatography in n-butanol/acetic acid/

water (4:1:5, v/v). After drying, high voltage electrophoresis

was performed in the second dimension in a buffer composed of

pyridine/acetic acid/water (1:10:189, v/v), pH 3.65, at 2000

volts for one hour. The peptides were stained with 0.05% ninhydrin

in ethanol and heated to 70° for 20 minutes.

Analytical Methods and Physical Measurements

Procedures for the quantitative analysis of amino acids,

fatty acids, glucosamine, ash, phosphorus, nitrogen, carbon and

hydrogen were the same as described in a previous paper (18).

Analytical ultracentrifugation, agarose electrophoresis and

infrared spectroscopy of "conjugated" protein and/or its fragments

were carried out according to the procedures described previously

(18).

Immunological Methods

Preparation of antibodies to "conjugated" protein and

tryptic and pronase cores, and double diffusion and immunoelec-

trophoresis in 1% agarose gel were performed according to the

procedures previously described (18).

Antigens and the homologous antibodies are symbolized by

the same letters; capital letters designate antigens and small

letters antibodies:

Antigens		Antibodies	
"Conjugated" protein	G	Anti-"conjugated" protein	g
C.P. tryptic core	H	Anti-tryptic core	h
C.P. pronase core	I	Anti-pronase core	i

Lethality for Mice (LD_{50})

Two different strains of white male mice (Swiss-register,

random bred, Pel-Freeze, Bio-Animals, Inc., Rogers, Arkansas,

and Balb-C inbred mice, Texas Inbred Mouse Co., Houston, Texas)

weighing 17-21 g were used for toxicity studies. Each group

consisted of six mice. The various preparations were tested

at least two times each. The substances, suspended either in

0.9% NaCl or in 0.05 M Tris buffer containing 0.5% SDS, pH 7.6,

were injected intraperitoneally. Control animals were injected

with the same buffer system. Deaths occurring within 7 days

were registered. The LD_{50}-values were calculated according to

Cornfield and Mantel (21). Categories of toxicity were assigned

according to Loomis (22).

RESULTS

Isolation of "Conjugated" Protein and Corresponding

Tryptic and Pronase Cores

The endotoxin complex (LPS-U-S) isolated by trichloroacetic

acid extraction of S. marcescens 08 contained approximately 20%

lipids extractable by chloroform-methanol. "Conjugated" protein

isolated from the defatted endotoxin complex (LPS-Ud-S) by

hydrolysis with 1% acetic acid represented 36% of the starting

material (Fig. 1). The acetic acid hydrolysate was extracted

with chloroform and the small amount of material present in the

chloroform phase was submitted to hydrolysis with 0.1 N HCl;

the hydrolysate contained only traces of fatty acids. The

unsaponifiable material was not investigated.

The polysaccharide moiety, accounting for 60% of the endotoxin

complex, was separated by dialysis into an inner and outer

dialysate. The non-dialyzable fraction, representing approximately

52% of the polysaccharide moiety, contained 41.8% C, 6.2% H,

3.3% N and less than 0.4% P. Amino acid analysis revealed only

trace amounts of amino acids and 38.4% glucosamine. Results of

quantitative gas-liquid chromatography (23) indicated that the

inner dialysate contained glucose, galactose and glucosamine in

a molar ratio 1:1:2. This fraction, representing most probably

the O-specific "side chain" of the polysaccharide moiety, gave

a single precipitin line with antibodies to LPS-Ud-S (18). The

outer dialysate contained 2.2% P, 5.6% N and heptose as the most

characteristic sugar component (23). Since heptose is the typical

component of the polysaccharide "core" (17), the outer dialysate

represents a partially degraded "core" portion of the poly-

saccharide moiety. Both fractions were soluble in distilled

water.

The tryptic core represented 75% of "conjugated" protein

and the pronase core 60% of tryptic core or approximately 45%

of "conjugated" protein.

Physical-Chemical Characterization of "Conjugated" Protein

and Corresponding Tryptic and Pronase Cores

The "conjugated" protein and both cores were soluble in

ammonium carbonate buffer, pH 8.6, and precipitable by addition

of acetic acid to pH 3.5-3.8. However, after repeated lyophiliza-

tions the protein preparations were soluble only in 0.05 M Tris

buffer containing 0.2% SDS, pH 7.6.

The "conjugated" protein exhibited a single, symmetrical

peak in the analytical ultracentrifugal with s_{obs} = 2.4S (Fig. 2).

In agarose electrophoresis, "conjugated" protein, both

cores and the endotoxin complex (LPS-Ud) migrated as single

bands (Fig. 3).

Infrared spectra of "conjugated" protein and its tryptic

and pronase cores are shown in Fig. 4. Although spectra resemble

those of the "simple" protein and its cores (18, 19), the ν CH_3

and CH_2 absorption at 2960-2850 cm^{-1}, the ν C-O and γ OH

carbohydrate absorption at approximately 1060 cm^{-1} and the

ν P = O band at approximately 1230 cm^{-1} are more distinct in the

spectra of the "conjugated" protein and its tryptic and pronase

cores than in the spectra of "simple" protein and its corresponding

cores. In contrast to "simple" protein, the "conjugated" protein

exhibited a distinct ν C = O ester absorption which gradually

increased in the tryptic and pronase cores. The ester/amide I

ratios are shown in Table 1.

Immunological Properties of "Conjugated" Protein

and Its Tryptic and Pronase Cores

Like "simple" protein and its cores (18, 19), the "conjugated"

protein and the corresponding tryptic and pronase cores were

found to be immunogenic. The "conjugated" protein (G) exhibited

a single precipitin line against anti "conjugated" protein

serum (g) (Fig. 5, pattern 1). The tryptic core (H) and pronase

core (I) gave also single precipitin lines with antibodies to

"conjugated" protein (g) (Fig. 5, pattern 2), tryptic core (h)

and pronase core (i) (Fig. 5, pattern 3). The precipitin lines

of these three protein preparations showed complete fusion with antibodies to "conjugated" protein as well as with antibodies to pronase core (Fig. 5, patterns 2 and 3). Immunoelectrophoretic patterns of the tryptic (H) and pronase (I) cores show clearly that both fragments of "conjugated" protein exhibited single precipitin arcs (Fig. 6).

The "conjugated" protein (G), "simple" protein (A) from S. marcescens O8 and "simple" protein (B) from E. coli O 141K85(B) gave lines of total coalescence with antibodies to "simple" protein (a) from S. marcescens O8 (Fig. 7, pattern 1). A similar line of total fusion was also obtained in the reaction of "conjugated" protein (G) with antibodies to "conjugated" protein (g), "simple" protein (a), C.P. tryptic core (h) and C.P. pronase core (i) (Fig. 7, pattern 2). However, the precipitin line of "conjugated" protein with antibodies to endotoxin complex, LPS-Ud-S, (k) showed only partial identity with lines obtained with antibodies to various protein fragments.

Chemical Characterization of "Conjugated" Protein

and Its Tryptic and Pronase Cores

The "conjugated" protein and both cores contained fatty

acids, glucosamine and phosphate, the characteristic constituents

of lipid A, but not even traces of other sugars or lipids (Table 2).

A comparison of the percent chemical composition of "conjugated"

protein with that of "simple" protein (18) shows a greater content

of fatty acids, glucosamine and phosphate and a smaller content

of amino acids in the "conjugated" protein. Successive proteolytic

degradation of the "conjugated" protein with trypsin and pronase

resulted in a decreased content of amino acids and nitrogen and

an increased content of carbon, hydrogen, phosphate, glucosamine

and fatty acids. Pronase digestion resulted in more pronounced

change in the chemical composition than trypsin hydrolysis. The

molar ratio fatty acids/glucosamine/phosphate of the "conjugated"

protein and its cores (3:1:1) was higher than that of the "simple"

protein and its corresponding cores (2:1:1). Thus, the fatty

acid content of "conjugated" protein was higher than that of the

"simple" protein.

The amino acid composition of "conjugated" protein and its

tryptic and pronase cores is presented in Table 3. Aspartic acid

was the major amino acid and half-cystine was absent. Neither

glucosamine nor fatty acids were detected upon hydrolysis of the

tryptic and pronase peptides.

Gas-liquid chromatography of the fatty acids isolated from

"conjugated" protein and both cores revealed again that β-OH-myristic

acid was the major fatty acid (Table 4). Hydrogenation and

acetylation of the fatty acid methyl esters confirmed the presence

of unsaturated and hydroxy fatty acids.

Isolation of Lipid A from "Conjugated" Protein

Lipid A was isolated from "conjugated" protein by mild

hydrolysis with 0.1 N HCl according to the procedure used for

its isolation from "simple" protein (19). The infrared spectrum

of lipid A from "conjugated" protein (Fig. 4) was identical with

the spectrum of lipid A isolated from the lipopolysaccharide

fragment of endotoxin complex (24, 25). The ester/amide I ratio

of lipid A (0.96) was higher than that of the pronase core (0.86)

(Table 1). The glucosamine content of lipid A was 24%.

Comparison of the Chemical Composition of the

"Simple" Proteins and "Conjugated" Protein

Results shown in Table 5 point out clearly some differences

in the chemical composition of the "simple" proteins isolated

from S. marcescens 08 (PX-S) and E. coli O 141K85(B) and the

"conjugated" protein prepared from S. marcescens 08. The

"conjugated" protein contains a higher relative content of

glucosamine, fatty acids and phosphate and, therefore, a higher

content of lipid A than the "simple" proteins. On the other

hand, the "simple" protein PX-S seems to contain more lipid A

than PX-E.

Molar ratios of amino acids of "conjugated" and "simple"

proteins from S. marcescens were very similar, if not identical;

however, they differed to some extent from those of "simple"

protein from E. coli. Similarly, while two-dimensional patterns

of tryptic peptides of C.P. and PX-S were almost identical, they

differed in certain regions from that of PX-E (Figs. 8 and 9)

Toxicity Studies

The LD_{50} values of the endotoxin complex and its fragments

were influenced to a large extent by solubility of the substances

in the two different solvent systems used and by the strain of

mice tested (Table 6). Endotoxin complex, "conjugated" protein,

lipopolysaccharide and lipid A were highly toxic in inbred mice

when administered in Tris-SDS buffer. Removal of the polysaccharide

or protein moiety from the endotoxin complex decreased but did

not abolish the toxicity of the remaining fragments ("conjugated"

protein and lipopolysaccharide, respectively). Although the

"conjugated" protein exhibited a higher toxicity than the "simple"

protein, the latter should still be considered as moderately

toxic. However, removal of the "conjugated" protein from the

endotoxin resulted in the isolation of a non-toxic, partially

degraded polysaccharide moiety. Preparations solubilized in

Tris-SDS buffer displayed much higher toxicity than those suspended

in 0.9% NaCl. The 0.05 M Tris buffer containing 0.5% SDS, pH 7.6,

was not toxic for mice.

DISCUSSION

Chemical analysis of "conjugated" protein has confirmed

the assumption of Westphal and Lüderitz (15) that this fragment

of endotoxin complex consists of lipid A and protein. "Conjugated"

protein contained no other compounds except amino acids and

typical constituents of lipid A. "Simple" and "conjugated"

protein preparations differed in quantitative rather than

qualitative chemical composition. The relative content of lipid

A constituents of "conjugated" protein was greater than that of

"simple" protein. Like "simple" protein (19), lipid A was firmly

bound to the protein moiety of "conjugated" protein and was

extracted by chloroform only after hydrolysis of the protein

preparation by 0.1 N HCl. Similarity of the molar ratios of

amino acids, distribution of tryptic peptides and immunological

properties demonstrated clearly the identity of the protein

moieties of "simple" and "conjugated" proteins. It has been

suggested recently (17) that the presence of lipid A in the

"conjugated" protein represents the only difference between

these two protein fragments. However, results of our studies

show that "conjugated" and "simple" proteins are not distinct

by the presence or absence of lipid A, but rather by the presence

of a larger or smaller amount of lipid A linked to the protein

moiety. Accordingly, the "conjugated" protein consists of the

protein moiety linked to the entire lipid A, whereas in "simple"

protein the identical protein moiety is bound only to a fragment

of lipid A.

These unexpected results have raised intriguing questions

not only about the structure of lipid A, but also about the

differing effects of aqueous phenol and trichloroacetic acid on

the structure of endotoxin in general and or lipid A in particular.

The structure of lipid A is not yet fully understood. Westphal

and Lüdertiz (15) have considered lipid A as a single molecular

entity constituting an integral part of the endotoxin complex.

However, the fractionation of lipid A isolated from lipopolysac-

charides by mild acid hydrolysis disclosed a variety of compounds

such as free fatty acids and glucosamine on one hand, and unaltered

phosphorylated acylpolyglucosamines of different chain length on

the other (25-28). Although the breakdown products resulting from

acid hydrolysis may be visualized as parts of a single structural

unit of lipid A, it has also been suggested that lipid A may

represent a mixture of related but not identical structures

derived from different subunits of the complex endotoxin structure

(29). Our conclusion supporting the concept of lipid A as a

single structural entity is based on following experimental

facts: 1) after 1% acetic acid hydrolysis of the endotoxin

complex, the lipid A constituents were detected only in the

"conjugated" protein, 2) lipid A constituents could not be ex-

tracted by organic solvents from electrophoretically- and

immunochemically-homogeneous "conjugated" protein, 3) "conjugated"

protein contained no sugars other than glucosamine; and 4)

lipid A constituents were absent in the mixtures of free amino

acids and/or peptides released by proteolytic degradation of

"conjugated" protein by trypsin and pronase. Nevertheless, it

is difficult to visualize lipid A as a simple linear structure

consisting of a phosphorylated polyglucosamine chain with ester-

and amide-bound fatty acids. More likely, lipid A possesses a

branched structure consisting of several polyglycosamine chains

of different length linked together through phosphodiester linkages.

Although the predominant bond between O,N-acylated glucosamine

phosphate units seems to be of glycosidic nature (17, 24, 25,

28, 30), a phosphodiester linkage even within a single poly-

glucosamine should not be excluded (25, 31). In contrast to the

stable glucosamine glycosides, the phosphodiester linkages are

very labile to mild acid hydrolysis (32-34). As a result of

mild acid hydrolysis of phosphodiester linkages between or within

polyglucosamine chains, the phosphate groups should remain linked

to glucosamine especially as very stable monoesters in positions

C_4 and C_6 (30, 35). Mild acid hydrolysis used for the isolation

of lipid A may cleave the phosphodiester linkages and result

in breakdown products of different sizes.

Since its introduction by Westphal et al. (36), aqueous

phenol extraction has been one of the most frequently used

methods for the isolation of lipopolysaccharides. It has been

assumed that this procedure causes primarily a dissociation of

endotoxin into the protein and lipopolysaccharide fragments

with little, if any, cleavage of covalent linkages. Proteins,

indeed, do not seem to be altered by phenol treatment as shown

by the unchanged physical-chemical properties and enzymatic

activity of ribonuclease (37). Tsang (25) studied the effect

of aqueous phenol treatment on the chemical composition of

lipopolysaccharides from $\underline{S}$. marcescens 08 and reported a markedly

decreased fatty acid and glucosamine content of a lipopolysac-

charide preparation submitted to repeated extraction by aqueous

phenol. He concluded from these results that phenol treatment

caused cleavage of ester- and amide-bound fatty acids and at

least a partial degradation of lipid A.

One can summarize the various effects of phenol (pKa = 10.0),

acetic acid (pKa = 4.76) and trichloroacetic acid (pKa = 0.08)

on whole endotoxin in the following manner. Although phenol

and acetic acid were weaker acids than trichloroacetic acid,

at high temperatures (70° and 90°, respectively) they cause a

selective partial cleavage of endotoxins. Phenol cleaves some

ester- and amide-bound fatty acids and dissociates the protein

moiety by splitting a particular (phosphodiester?) linkage within

the lipid A. Acetic acid, on the other hand, seems to break

specifically the linkages between the lipid A and the polysac-

charide moiety as well as the linkages between the "side chain"

and the "core" portions of the polysaccharide. The isolation

of a relatively intact endotoxin complex by trichloroacetic acid

is due most probably to the fact that the extraction of bacterial

cells is carried out at low temperature (4°). It has been shown,

however, in a preceding paper of this series (18) that even at

that temperature the endotoxin complex contains degradation frag-

ments such as "simple" protein, side chain and free fatty acids.

The importance of the reaction temperature is best illustrated

by the finding that cold phenol extraction does not dissociate

the protein moiety from the endotoxin complex (36). The effec-

tiveness of phenol may be due also to its property as an excellent

solvent for proteins (37); by unfolding the entire endotoxin

structure as a solvent it may cause the maximal exposure of

susceptible linkages to its action as a reagent.

The difference between the approximate molar ratios of

fatty acids/phosphate/glucosamine of "conjugated" protein and

its tryptic and pronase cores (3:1:1) and "simple" protein

and its corresponding cores (2:1:1) may be explained now by

the enhanced cleavage of ester bound fatty acids by phenol

treatment. Although it has been assumed (36) that phenol causes

a complete separation of the protein moiety from the lipopoly-

saccharide fragment of the endotoxin complex, results of our

studies indicate that hot phenol treatment cleaves the endotoxin

within the lipid A portion; the resulting "simple" protein

consists then of the protein moiety still linked to a fragment

of lipid A.

There are very few reports describing the chemical composition

of well defined "simple" and "conjugated" proteins from other

Gram-negative bacteria. Homma (38) isolated the protein moiety

of endotoxin from Pseudomonas aeruginosa and found that it contained

13.1% N, 1.6% P and 2.3% galactosamine. Alkaline ethanol treatment

resulted in the isolation of a protein with 15.4% N. Fatty acids

were not determined. Since the protein moiety contained 11%

glucose, it is very difficult to classify it either as a "simple"

or as a "conjugated" protein. Jenkin and Rowley (39) isolated

toxic proteins from Vibrio cholerae and Water vibrios. Toxic

protein accounted for the major portion of the toxicity of the

whole organism. Immunological and chemical data suggested that

the toxic protein was identical with the protein moiety of the

Boivin antigen. Toxic protein from Vibrio cholerae contained

13.8% N and 1.5% P; no values for carbohydrate or fatty acids

were reported. Previous studies on the isolation of "conjugated"

protein from Brucella melitensis (40), Shigella dysenteriae

(1, 41) and Shigella paradysenteriae (Flexner) (5) showed that

these preparations had nitrogen values (9-11%) higher than that

of the "conjugated" protein but still lower than that of the

"simple" protein from S. marcescens 08. It remains for future

studies to establish whether this difference in nitrogen content

reflects a lower content or an incomplete removal of lipid A

from endotoxins of other species of Gram-negative bacteria.

Antibodies prepared against "conjugated" protein and its

tryptic and pronase cores showed the presence of a common antigenic

determinant in all three protein preparations. Several investigators (3-5, 41) have already shown that "conjugated" proteins treated with trypsin retained their immunogenic properties. It appears that the antigenic site is located most probably on the protein moiety close to the lipid A.

Since toxicity represents one of the most characteristic biological properties of endotoxins, the search for a toxic principle or site in the complex has been based on two alternative hypotheses proposing either the presence of a toxic constituent in or a toxic "conformation" of endotoxin structure (29). Goebel and his coworkers (4, 5) suggested that a toxic "factor T" present in the so-called "toxic" protein preparation (a preparation equivalent to "conjugated" protein) was neither a protein nor a polysaccharide. Westphal and Lüderitz (15) proposed later that the toxic "factor T" was identical with lipid A, and detoxification studies (29) by partial alkaline hydrolysis showed that the presence of ester-bound fatty acids in endotoxins was essential

for eliciting toxic reactions. The polysaccharide moiety was found not to be essential for the toxicity, because endotoxin preparations from R-mutants of various Salmonella strains lacking the entire polysaccharide moiety still retained potent toxic properties (42-44). On the other hand, Ribi and his coworkers (45) isolated from S. enteritidis a highly toxic endotoxin characterized by a low fatty acid content and suggested (46) that a macromolecular complex of critical size is a major requirement for toxicity and other biological properties of endotoxins.

Results of the present investigation show clearly that endotoxin and its fragments dissolved in Tris-SDS buffer exhibit much higher toxicity than the same fractions administered as saline suspensions. It appears that SDS, by decreasing the particle size of the endotoxin fractions, had an enhancing rather than a diminishing effect on their toxic properties; similarly, McIntire et al. (46) have shown that disaggregation of a lipo-polysaccharide preparation from E. coli K-235 by SDS did not

decrease pyrogenicity in the rabbit. The whole endotoxin complex,

LPS-Ud-S, was the most toxic preparation. The removal of the

polysaccharide moiety affected the toxicity of the remaining

fragment more than a corresponding cleavage of the protein moiety;

however, both the "conjugated" protein (LD_{50} = 22 mg/kg) and the

lipopolysaccharide (LD_{50} = 12 mg/kg) should be considered as

highly toxic fragments. Although "simple" protein, containing

only a small part of lipid A, was less toxic than "conjugated"

protein, it still displayed a moderate toxicity. It was shown

for the first time that lipid A, due to an increased solubility

in SDS, is a highly toxic fraction (LD_{50} = 14 mg/kg). These

results demonstrate clearly the importance of the lipid A for

the toxic properties of endotoxins and support strongly the

proposal by Westphal and Lüderitz (5, 42) that lipid A is the

toxic factor of endotoxins.

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Table 1

Ester/Amide I Ratios of "Conjugated Protein", Tryptic

and Pronase Cores and Lipid A

	Length of Amide I Band in mm	Length of Ester Band in mm	Ester/Amide I Ratio
"Conjugated" Protein	84	57	0.67
Tryptic core	82	61	0.74
Pronase core	64	55	0.86
Lipid A	65	63	0.96

Table 2

Elementary Analysis and Chemical Composition of "Conjugated" Protein, Tryptic Core and Pronase Core

	C %	H %	N %	PO_4 %	Glucosamine %	Fatty Acids %	Amino Acids %	Recovery[a] %	Ash %
"Conjugated" Protein	54.74	8.26	6.30	4.98	9.4	46.0	35.3	95.8	0.46
CP tryptic core	54.86	8.37	5.26	5.52	11.7	49.6	30.7	97.5	None or Trace
CP pronase core	57.39	9.23	3.45	6.48	18.1	53.2	12.9	90.7	None or Trace

[a]Recovery is calculated from phosphate, glucosamine, fatty acids and amino acids.

Table 3

Amino Acid Composition of "Conjugated" Protein,

Tryptic Core and Pronase Core

	"Conjugated" Protein		Tryptic Core		Pronase Core	
	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100
Lysine	135	1.97	82	1.20	73	1.07
Histidine	31	0.48	35	0.53	15	0.23
Arginine	82	1.43	48	0.84	42	0.73
Aspartic acid	457	6.08	328	4.36	197	2.62
Threonine	157	1.87	108	1.29	53	0.63
Serine	212	2.23	139	1.46	108	1.13
Glutamic acid	327	4.81	212	3.12	120	1.77
Proline	85	0.98	59	0.69	55	0.63
Glycine	332	2.49	294	2.21	203	1.52
Alanine	315	2.81	227	2.02	140	1.25
1/2 Cystine	-	-	-	-	-	-
Valine	202	2.37	153	1.79	83	0.97
Methionine	45	0.67	39	0.30	-	-
Isoleucine	95	1.25	68	0.90	42	0.55
Leucine	224	2.94	158	2.07	75	0.98
Tyrosin	156	2.83	140	2.47	51	0.92
Phenylalanine	134	2.21	99	1.63	54	0.89

Table 4

Percent Fatty Acid Composition of "Conjugated" Protein and

Its Tryptic and Pronase Cores

	"Conjugated" Protein	Tryptic Core	Pronase Core
C_{12}	4.0	2.1	5.1
C_{14}	17.0	17.2	21.8
C_{14-1}	-	-	2.1
Unknown	11.6	11.6	7.5
C_{16}	13.0	13.6	12.0
C_{16-1}	5.4	6.2	4.1
Unknown	9.6	5.2	4.6
$C_{14}-\beta-OH$	39.5	41.4	41.8

Table 5

Chemical Composition of "Conjugated" Protein, PX-S and PX-E

Constituents	"Conjugated" Protein	PX-S	PX-E
	%	%	%
Amino acids	35.26	72.89	84.42
Glucosamine	9.35	2.87	1.31
Fatty acids	46.08	10.10	5.98
PO_4^{3-}	4.98	2.01	1.59

Table 6

LD_{50} of Intact Endotoxin and Its Various

Fragments from S. marcescens 08

	Random bred Swiss-regular Mice		Inbred Balb-C Mice	
	In Tris-SDS	In Saline	In Tris-SDS	In Saline
	mg/kg		mg/kg	
Endotoxin complex (LPS-Ud-S)	7.5	50.0	1.3	13.4
"Conjugated" Protein	37.5	197.5	22.3	> 75.0
"Simple" Protein (PX-S)	55.0	n.d.[a]	27.5	>100.0
Protein-free Endotoxin (LPS-A)	n.d.	62.5	12.1	39.9
Lipid A	n.d.	n.d.	14.2	n.d.
Polysaccharide side chain	non-toxic		non-toxic	

[a]Not determined.

LPS-Ud-S

1. Hydrolysis with 1% acetic acid;
   4 hrs, 90°C

2. Storage at 4°C for 1 hr

3. Centrifugation at 17,300 x g for
   20 minutes at 10°C

Sediment

1. Dissolved in 0.02 M
   $(NH_4)_2CO_3$, pH 8.6

2. Precipitation with
   CH_3COOH at pH 3.5

3. Centrifugation

Sediment

1. Washing for 24
   hrs with dis-
   tilled water

2. Centrifugation

3. Lyophilization

"Conjugated" protein

Supernate

Supernate

Extraction with $CHCl_3$

Aqueous phase   Lyophilization

"Degraded" Polysaccharide

Dialysis against
distilled water

Inner dialysate

"Side chain"

Chloroform phase

Outer dialysate

"Core"

Figure 1. Isolation of "conjugated" protein from LPS-Ud-S.
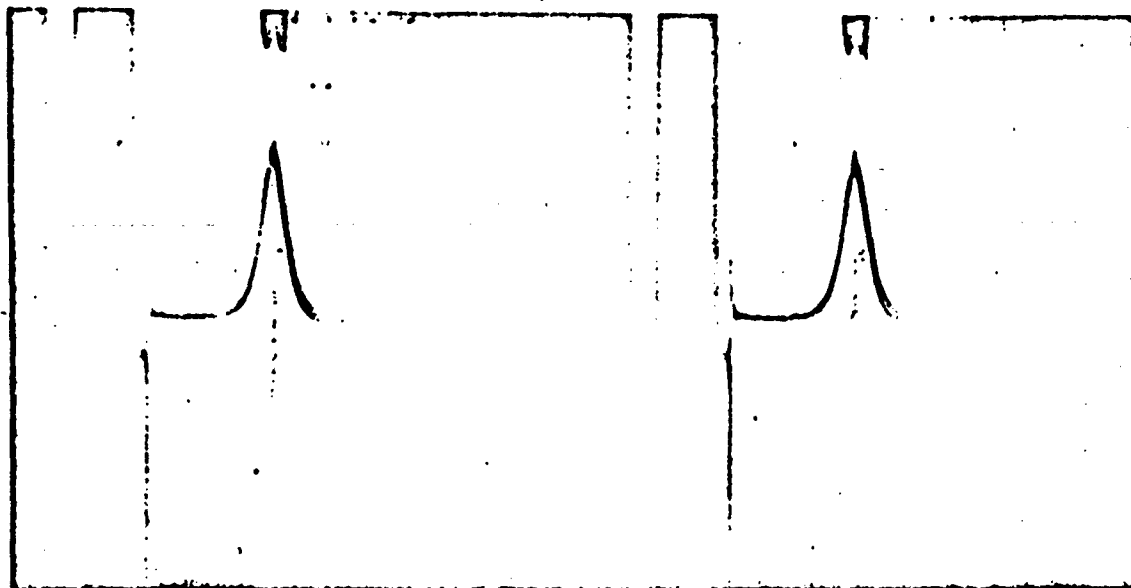
Figure 2.   Sedimentation velocity pattern of "conjugated" protein.
"Conjugated" protein (6 mg/ml) dissolved in 0.05 M Tris-Cl
buffer, pH 7.6, containing 0.2% SDS, solvent density 1.004 g/ml,
was centrifuged in a synthetic boundary cell at 52,640 rpm at
25 . Exposures were taken from left to right at 4 minute
intervals for a total run of 64 minutes. Photographs shown
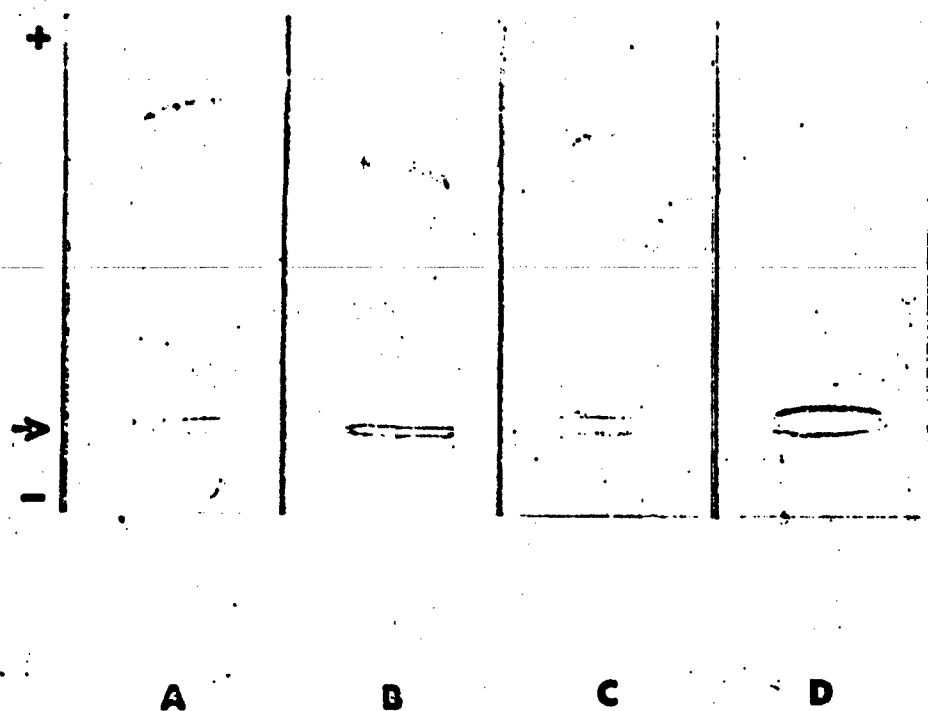were obtained at 12 and 32 minutes after reaching speed.

Figure 3. Agarose electrophoresis of LPS-U-S (A), "conjugated" protein (B), tryptic core (C) and pronase core (D). Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer. Electrophoresis was performed in 1% agarose gel using Veronal buffer, pH 8.6, ionic strength 0.05 for 40 minutes.
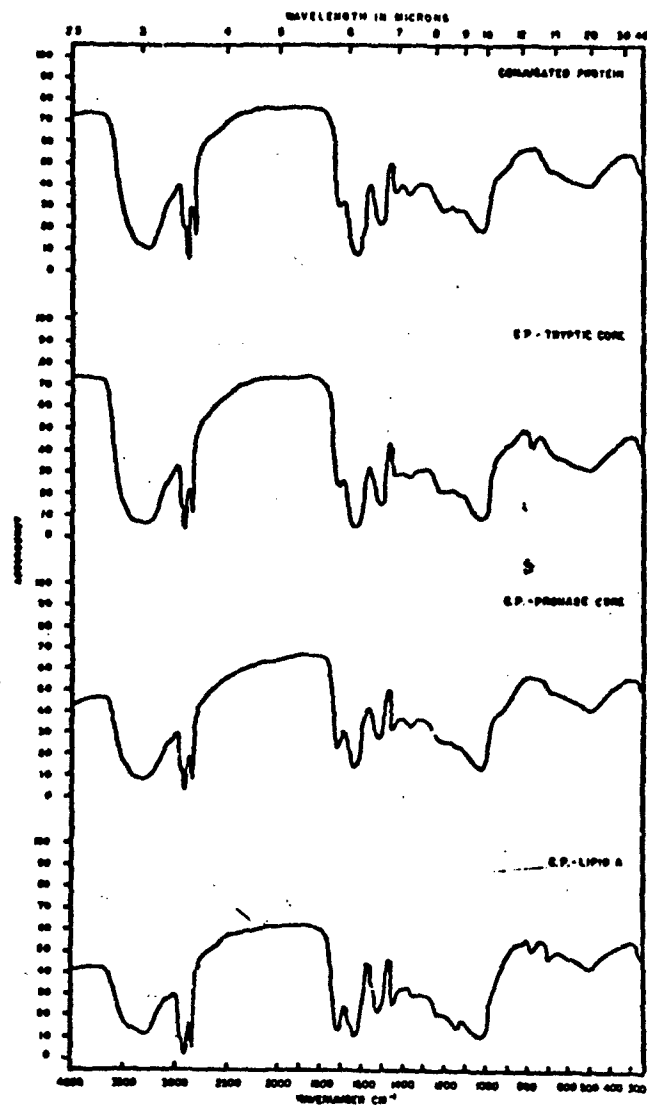
Figure 4. Solid infrared spectra of "conjugated" protein, tryptic and pronase cores and lipid A.
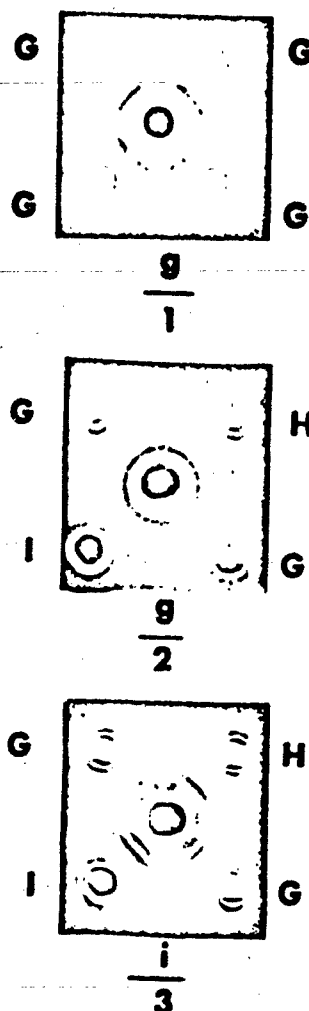
Figure 5. Immunodiffusion patterns of "conjugated" protein (G), tryptic core (H) and pronase core (I). Central wells contain antibodies to "conjugated" protein (g) and to pronase core of "conjugated" protein (i).
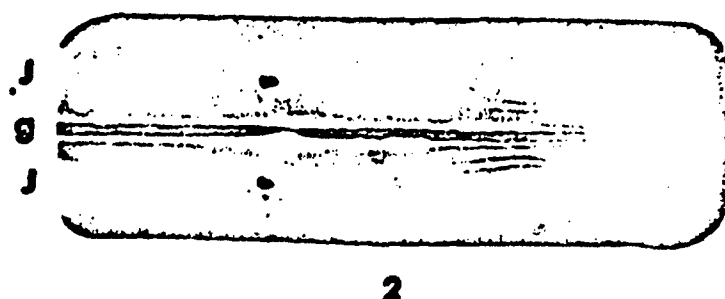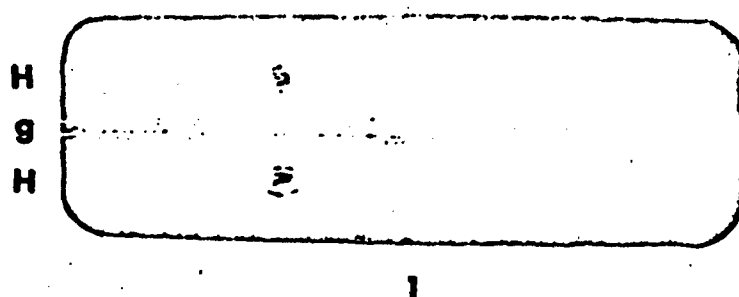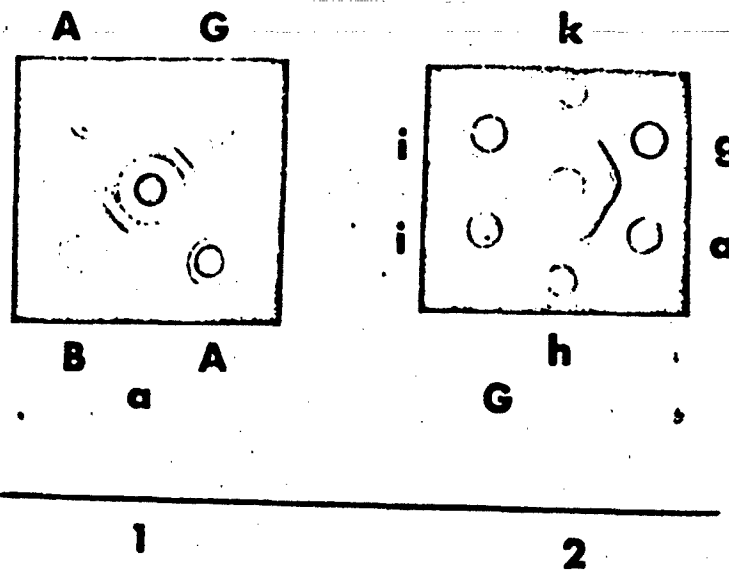
Figure 6. Immunoelectrophoresis patterns of tryptic core (H) and pronase core (I). The central troughs in both patterns contain anti-bodies to "conjugated" protein.

Figure 7. Immunodiffusion patterns of PX-S (A), PX-E (B) and "conjugated" protein (G). The central well in pattern 1 contains antibodies to PX-S (a). The outer wells in patters 2 contain antibodies to PX-S (a), LPS-U-S (k), "conjugated" protein (g), tryptic core of "conjugated" protein (h), and pronase core of "conjugated" protein (i).

Figure 8. Peptide mapping of tryptic peptides of "conjugated" protein (upper pattern) and PX-S (lower pattern). Experimental conditions are described in the Materials and Methods.

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13. ABSTRACT

"Conjugated" protein isolated from the endotoxin complex of Serratia marcescens 08 by 1% acetic acid hydrolysis was characterized by analytical ultracentrifugation, electrophoretic and immunological properties and chemical analysis. The chemical composition of "conjugated" protein and other degraded fragments of endotoxin showed that all characteristic constituents of lipid A were present exclusively in "conjugated" protein. Successive treatment of "conjugated" protein with trypsin and pronase resulted in the isolation of corresponding tryptic and pronase resulted in the isolation of corresponding tryptic and pronase cores which were characterized by an increased content of lipid A constituents. Lipid A was separated as an entity from the "conjugated" protein only after hydrolysis with 0.1 N hydrochloric acid. This result confirms our previous proposal that lipid A is covalently linked to the protein moiety in whole endotoxin of S. marcescens 08. "Conjugated" protein isolated by acetic acid hydrolysis and "simple" protein prepared by hot aqueous phenol treatment of whole endotoxin differ from one another not by the presence or absence of lipid A but rather by the presence of the entire lipid A moiety in the "conjugated" protein and only a fragment of lipid A in the "simple" protein. "Conjugated" protein and its tryptic and pronase cores were immunogenic and possessed a common antigenic determinant with "simple" protein and its corresponding cores. Toxicity studies revelaed that both "conjugated" and "simple" proteins retained to different degrees the toxic properties of whole endotoxin. Sodium dodecyl sulfate had an enhancing rather than diminishing effect on the toxicity of endotoxin and its various fragments.

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